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Ammonium (methylammonium) is the co-repressor of nitrate reductase in *Chlamydomonas reinhardii*

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Methylammonium cannot be used as a nitrogen source by the green alga *Chlamydomonas reinhardii* and, like ammonia, caused the repression of nitrate reductase without affecting the photosynthetic activity. Glutamine synthetase catalyzed the conversion of methylammonium to a single product, identified as γ-*N*-methylglutamine, which accumulated in the cells. Derepression of nitrate reductase was accompanied by a decrease in the intracellular concentration of methylammonium and a concomitant accumulation of γ-*N*-methylglutamine inside the cells. These facts strongly suggest that ammonium (methylammonium) per se, and not a product of its metabolism, is the co-repressor of nitrate reductase in *C. reinhardii*

Nitrate reductase *Ammonium-repression* *Methylammonium* Chlamydomonas reinhardii

1. INTRODUCTION

In photosynthetic organisms, nitrate reductase (EC 1.6.6.1–3), the key enzyme of the nitrate assimilatory pathway, is an adaptive enzyme, almost absent in cells grown in the presence of ammonium [1–3].

At present it is unresolved whether ammonium per se or, by contrast, a product of its metabolism, is the true co-repressor of the enzyme [2–5]. Most data suggesting this latter possibility have been obtained from experiments carried out with the specific inhibitor of glutamine synthetase (EC 6.3.1.2) MSX [6–10] or from kinetic results on ammonia inhibition [4] or with fungal mutants deficient in glutamine synthetase [5,11]. Methylammonium mimics all the effects of ammonium on nitrate assimilation [12–14], and in different organisms enters the cells by the same transport system of ammonia [15–19]. Very recently it has been demonstrated that glutamine and its analogs like MSX inhibited the ammonia transporter in different non-photosynthetic and photosynthetic bacteria [20–22], which forces to reevaluate the conclusions reached from the use of such inhibitors.

Here we propose, on the basis of experiments with methylammonium, that ammonium per se, and not a product of its metabolism, is the true co-repressor of nitrate reductase in the green alga *Chlamydomonas reinhardii*.

2. MATERIALS AND METHODS

C. reinhardii 21*gr* (+) (a gift from Dr R. Sager, Hunter College, New York) was grown in ammonium liquid media [23]. Nitrate reductase activity was assayed in situ in cells permeabilized with toluene [24]. The biosynthetic activity of glutamine synthetase was assayed by phosphate release [25] in crude extracts obtained as in [23]. A unit of enzymatic activity is defined as the amount of enzyme which catalyzes the transformation of 1 μmol substrate/min.

Nitrite was assayed by the diazotization method [26], and phosphate by the phosphomolybdate assay [27]. Chlorophyll was estimated as in [28] and protein as in [29].

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Abbreviation: MSX, L-methionine-DL-sulfoximine

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In radioactive studies cells were cultured with 1 mM $^{14}CH_3NH_3^+$ (5 mCi/mol, Amersham International) for the appropriate time, harvested by centrifugation at 23000 × *g* for 10 min, and washed with 50 vols ice-cold water. Chromatography of crude extracts was performed on silica gel plates (5 × 20 cm, 0.25 mm layer thickness) with methanol–water–acetic acid (60:38:2, v/v) [17]. Radioactivity of 0.5-cm strips scraped off the thin-layer plates was measured with a liquid scintillation β-counter KONTRON. Cold methylamine (R_F = 0.50) was co-chromatographed with samples. Plates were developed at 80°C after spraying them with a solution 0.25% (w/v) of ninhydrin in acetone.

3. RESULTS AND DISCUSSION

The ammonium analog methylammonium can be used as nitrogen source by different fungi and methylotrophic bacteria [12,15,30] but not by other types of photosynthetic [18,31,32] and non-photosynthetic [33,34] bacteria. *C. reinhardii* cannot utilize methylammonium (0.5–10 mM) as nitrogen source either. In the presence of methylammonium the alga was incapable of assimilating nitrate because, under such conditions, nitrate reductase activity was lacking (not shown).

Methylammonium entered and accumulated inside the cells at a rate about 20 μmol·mg chlorophyll^{-1}·h^{-1}. Since *C. reinhardii* cannot metabolize methylammonium, we have identified the accumulated products resulting from $^{14}CH_3NH_3^+$ by means of thin-layer chromatography. Only two peaks of radioactivity were found (fig.1A). One of these corresponded to an R_F = 0.50 coincidental with that of cold methylammonium used as marker, and the other one (R_F = 0.68–0.70) corresponded to γ-*N*-methylglutamine, a metabolite derived from methylammonium and L-glutamate found in certain bacteria [17,33,34].

Methylammonium acted as a substrate of glutamine synthetase of *C. reinhardii*. When the biosynthetic activity of extracts was assayed using methylammonium, a specific activity of 17 munits/mg protein (corresponding to a 16% of the activity found in the standard assay with ammonium [25]) was found. As expected, 1 mM

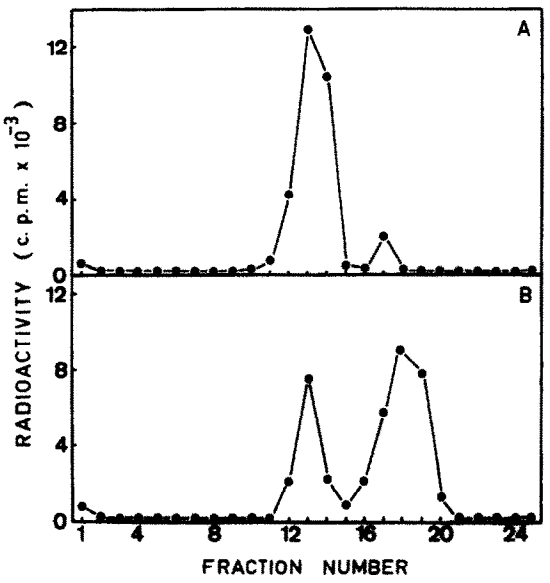

Fig.1. Chromatographic profile of $^{14}CH_3NH_3^+$ transformation products in *C. reinhardii*. (A) Cells (30 μg chlorophyll/ml) placed 2 h in 1 mM $^{14}CH_3NH_3^+$ (5 mCi/mol). (B) Cells as in A, washed and derepressed with 3 mM KNO_3 for 1.5 h. Each fraction corresponded to strips 0.5 cm width of the thin-layer plate. More details are given in section 2.

MSX added to the reaction mixture totally suppressed biosynthetic activities of glutamine synthetase with either ammonia or methylammonium leading to formation of glutamine or γ-*N*-methylglutamine, respectively (not shown). These results indicate that γ-*N*-methylglutamine is the unique product of enzymatic conversion of methylammonium catalyzed by glutamine synthetase in *C. reinhardii*. Biosynthesis of γ-*N*-methylglutamine from methylammonium by glutamine synthetase has been recently reported in cowpea *Rhizobium* sp. strain 32H1 [33] and *Azotobacter vinelandii* [34].

In the presence of nitrate, methylammonium was taken up by *C. reinhardii* cells and, after its exhaustion from the medium, nitrate reductase became derepressed. The same derepression was observed when cells placed in a methylammonium medium were washed and transferred to nitrate-containing media. In both cases a significant depression of intracellular levels of methylammonium and a parallel increase in those of γ-*N*-methylglutamine were found (fig.1B). This clearly

demonstrates that the intracellular levels of methylammonium, and not those of γ-*N*-methylglutamine, are responsible for the nitrate reductase repression. Methylammonium acted on nitrate reductase synthesis because of its structural similarity with respect to ammonium. An inhibitory effect of methylammonium on photophosphorylation, as reported in other photosynthetic organisms [14,35], must be ruled out since 4 mM methylammonium or ammonium did not impair photosynthetic oxygen evolution during 3 h in *C. reinhardii* cells placed in Mops–KOH buffer, pH 7.0 (not shown).

Data shown in fig.2 reinforce the conclusion that γ-*N*-methylglutamine is not involved in nitrate reductase repression in *C. reinhardii*. The glutamine analog accumulated inside the cells placed in media containing methylammonium. However, when methylammonium was eliminated from the media and cells were transferred to nitrate-containing media, nitrate reductase was derepressed to the same extent, irrespective of the time the cells had been previously kept in repression media. These changes were accompanied by a decrease in the intracellular methylammonium concentration (fig.1B).

In fig.3, the correlation between intracellular nitrate reductase and methylammonium and γ-*N*-methylglutamine levels are shown in more detail for a 3-h experiment. Similar results were obtained in experiments protracted to 12 h. Thus, our results demonstrate that ammonium (methylammonium) and not any product of its metabolism is responsible per se for repression of nitrate reductase of *C. reinhardii*. Our data can be explained by assuming that inside the cells subjected to this type of control an intracellular threshold level of ammonia exists above which this compound behaves as a co-repressor of nitrate reductase and under which the enzyme synthesis remains unaffected. It explains the 5-h lag needed in *C. reinhardii* to observe, in the presence of MSX, the disappearance of ammonia repression [8,9], since MSX first caused a rapid increase of intracellular levels of ammonia which is subsequently released to the medium prior to the synthesis of nitrate and nitrite reductases [36]. It would also explain satisfactorily why mutants of *Aspergillus nidulans* altered in their ammonium transporter did not exhibit repression of enzymes usually repressible by ammonium or methylammonium [12,16,37], since a low uptake rate of ammonium (methylammonium) can lead to intracellular concentrations of repressor under the threshold level, although in this particular case a regulatory role of some ammonium derivative cannot be ruled out.

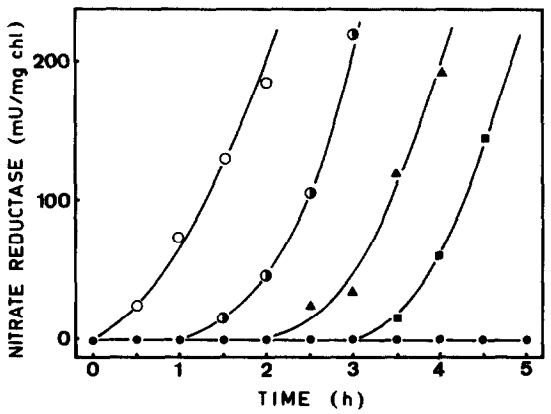

Fig.2. Derepression of nitrate reductase from *C. reinhardii* cells preincubated with methylammonium. Cells were placed in media 5 mM in methylammonium (●). After 0 (○), 1 (◑), 2 (▲) or 3 h (■) of incubation, the cells were washed with distilled water and transferred to media 3 mM in KNO_3. Nitrate reductase activity was assayed in situ in aliquots of cells subjected to different preincubation times.

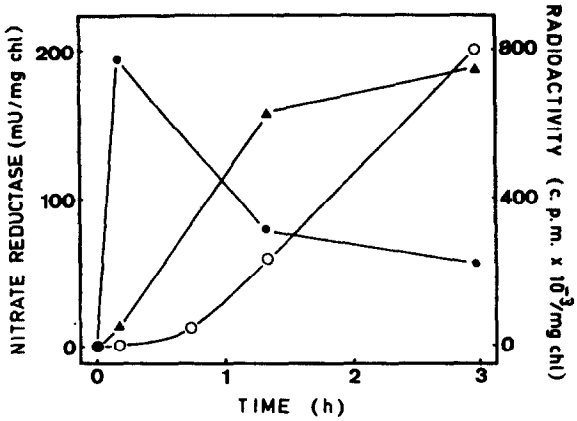

Fig.3. Time course of *C. reinhardii* nitrate reductase derepression in the presence of methylammonium. Cells (20 μg chlorophyll/ml) were incubated with 0.1 mM $^{14}CH_3NH_3^+$ (50 mCi/mmol). At the indicated times, nitrate reductase activity (○) was measured and intracellular radioactivity due to methylammonium (●) and γ-*N*-methylglutamine (▲) was determined by chromatography as described in section 2.

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